



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : C12N 15/12, C07K 14/705		A2	(11) International Publication Number: WO 97/20045
			(43) International Publication Date: 5 June 1997 (05.06.97)
(21) International Application Number: PCT/US96/18175		(81) Designated States: AL, AM, AU, BB, BG, BR, CA, CN, CZ, EE, FI, GE, HU, IL, IS, JP, KG, KP, KR, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 8 November 1996 (08.11.96)			
(30) Priority Data: 60/006,782 15 November 1995 (15.11.95) US 08/559,524 15 November 1995 (15.11.95) US			
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**(54) Title: P2u2 PURINERGIC RECEPTOR**

huP2U1 bovP2Y1 huP2U2	MAADP TEVMPAVP GIG	----- NGTDTAFLAD -----	----- PGSPGNSIV -----	GRNDLFLN PNAACK NHLAAEA	GTWDGDELGY TSTAAVASPF -----	RCRFNE-DFK KCALTKTGFO -----	32 50 23
huP2U1 bovP2Y1 huP2U2	YVLDPVSGV FYYPAVAIL -YLSIFG	VCGLDCLF VFDEFLGG EFVAVLGG	VGLYFLCR YAIMHVFHR MVVGYIFS	PLASTIN PLGSSIV PLGSSNII	PLGSSIV PLGSSIV PLGSSIV	PLGSSIV PLGSSIV PLGSSIV	82 100 72
huP2U1 bovP2Y1 huP2U2	AASVILNRY VLGGLAFY LCLGFLPS	MARGDHPFS FNKTDHIFG HAN-GNLYG	TYVCLVFL DAVCKLOFI DVAQTSNV	FYTLRQFT FHMVLEST FHMVLEST	PLGSSIV PLGSSIV PLGSSIV	PLGSSIV PLGSSIV PLGSSIV	132 150 121
huP2U1 bovP2Y1 huP2U2	LGVLRLRL SGAVYELKSL LTKYFIREH	RWGRARYRR GRLKKKNVY LLQKEFILL	PAGAVLVR ISVLAIMV ISVLAIMV	ACQAFVYFV VGISHLFYS LELLGLPLI	TTTSAR--GPL GTGIRKNKTI NPVI-TDNGT		180 200 170
huP2U1 bovP2Y1 huP2U2	RGVITAPEL DQVIRISPL EDVASSGD	FSRFVASSV LRSYFIFMC PYNLIISMIC	MLGLLPLF TYVAMPLF LTLGLPLF	AVILVCYVFL -LVLI-LGCV -LFVMCFYY	ARLLKPAYG GL-IVRALIY KIALFLKORN		230 246 218
huP2U1 bovP2Y1 huP2U2	TS6GLPRAKR KOLDNSPLRR QVDATPLPLE	SVKTEAVVL SYSLYTIIV SPNLISIMAV	AVPFLCFLF TQVSVYIF VGLVFLTFY	FATRTLYYSF PMYKTMRLA VMRNVRIRAS	-----SLDLS LDFQTPEN GLGSKQYCG		277 296 268
huP2U1 bovP2Y1 huP2U2	HTLNAPVAV AFNDRAVAT T-QVENSPLR	SVKTEAVVL SYSLYTIIV SPNLISIMAV	CLVFLPLF SCVFLPLF VGLVFLTFY	AVPFLCFLF FATRTLYYSF PMYKTMRLA	-----SLDLS LDFQTPEN GLGSKQYCG		326 346 316
huP2U1 bovP2Y1 huP2U2	ATPARRTLR EAL-NLG LQ-SFS	RRGDRTRR SKKQ-RR RWG-RR	IGDVGSSSE TLNIDSEFK LUS-FR	SRRTTESTPAG ----- -----	SENTKDRL ----- -----		375 373 335

**(57) Abstract**

Novel purinergic receptors are provided, having research, diagnostic and therapeutic applications. Also provided are nucleic acids encoding these receptors, as well as expression systems for their production.

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## P2U2 PURINERGIC RECEPTOR

Field of the Invention

The present invention relates to a new subtype of the P<sub>2</sub>-purinergic receptors, which is abundantly expressed in kidney and in many cell lines of megakaryocytic or erythroleukemic origin. Referred to herein as the P<sub>2U2</sub> receptor, this receptor is activated by ATP, ADP, UTP and UDP. The P<sub>2U2</sub> receptor can be used as a tool to screen or agonists and antagonists that can either stimulate or block receptor activation. Such compounds have therapeutic utility in treating (1) diseases that are caused by aberrant activation of this receptor, for example over stimulation or under stimulation of the receptor and (2) diseases whose symptoms can be ameliorated by stimulating or inhibiting the activity of the P<sub>2U2</sub> receptor.

The present invention also relates to the isolated entire human gene encoding the P<sub>2U2</sub> receptor, methods for the recombinant production of purified P<sub>2U2</sub> receptor proteins and the proteins made by these methods, antibodies against the whole P<sub>2U2</sub> receptor or regions thereof, vectors, nucleotide probes, and host cells transformed by genes encoding polypeptides having the P<sub>2U2</sub> receptor activity, along with diagnostic and therapeutic uses for these various reagents.

Background of the Invention

Purinergic receptors are cell surface receptors that interact with extracellular adenine or uridine nucleotides and nucleosides. These receptors are present throughout the central nervous system and peripheral tissues and play a role in numerous physiological responses.

The purinergic receptors are broadly divided into two major receptor types, P<sub>1</sub> and P<sub>2</sub>, which are defined by their level of interaction with the adenine nucleotides and nucleosides. Where P<sub>1</sub> receptors are activated by adenosine and exhibit a potency order of adenosine>AMP>ADP>ATP, P<sub>2</sub> receptors are activated by ATP, UTP, ADP or UDP and exhibit a potency of ATP≥ADP>AMP>adenosine. As more has become known about the purinergic receptors and the wide range of physiological responses in which

they play a role, the  $P_1$ - and  $P_2$ -type classifications were no longer sufficient to accurately portray this complex family of receptors. Therefore, receptor subtype categories have been developed. For example, the  $P_2$ -type purinergic receptors are now classified as  $P_{2Y}$ ,  $P_{2U}$ ,  $P_{2T}$ ,  $P_{2X}$  and  $P_{2Z}$ -subtypes. A review of the  $P_2$ -type purinergic receptors can be found in Harden, *et al.*, *Ann Rev Pharmacol Toxicol* (1995) 35:541-579.

Classification of the  $P_2$ -type purinergic receptors has been difficult because there are no published selective  $P_2$ -receptor antagonists and there are few ATP or ADP receptor-subtype specific agonists. In addition, it has been difficult to compare the relative order of potency of  $P_2$ -purinergic receptor agonists. Hence, this subtype has presented numerous challenges in the identification and characterization of its members.

#### Summary of the Present Invention

One aspect of the invention is an isolated and purified polypeptide comprising the amino acid sequence of Figure 1 (SEQ ID NO:2).

Another aspect of the invention is an isolated and purified nucleic acid sequence encoding for the  $P_{2U2}$  receptor.

Yet another aspect of the invention is an isolated and purified nucleic acid sequence comprising the nucleotide sequence of Figure 1 (SEQ ID NO:1).

#### Brief Description of the Drawings

Figure 1 is the DNA (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of the human  $P_{2U2}$  receptor.

Figure 2 is a comparison of the amino acid sequence of the human  $P_{2U2}$  receptor with the amino acid sequence of the human  $P_{2U}$  receptor (Parr, *et al.*, *Proc Natl Acad Sci USA* (1994) 91:3275-3279) (SEQ ID NO:3) and the bovine  $P_{2Y1}$  receptor (Henderson, *et al. BBRC* (1995) 212:649-656 (SEQ ID NO:4). The Parr  $P_{2U}$  receptor is referred to in Figure 2 as " $P_{2U1}$ ".

Figure 3 shows representative chloride currents obtained from oocytes injected with cRNA for the receptor and challenged with a variety of purinergic agonists (ADP, ATP, UTP, UDP).

### Description of the Preferred Embodiments

The present invention provides methods and materials useful in the regulation of the renal system in mammals. Recent studies provide evidence that extracellular  
5 nucleotides influence the renal microvasculature. See Inscho, *et al.*, *FASEB Journal* (1994) 8:319-328. The isolation, recombinant production and characterization of the purinergic receptor of the invention allows for the effective regulation of these functions.

Before proceeding further with a description of the specific embodiments of the present invention, a number of terms will be defined.

10 The terms "substantially pure" and "isolated" are used herein to describe a protein that has been separated from the native contaminants or components that naturally accompany it. Typically, a monomeric protein is substantially pure when at least about 60 to 70% of a sample exhibits a single polypeptide backbone. Minor variants or chemical  
15 modifications typically share approximately the same polypeptide sequence. A substantially pure protein will typically comprise over about 85 to 90% of a protein sample, preferably will comprise at least about 95%, and more preferably will be over about 99% pure. Purity is typically measured on a polyacrylamide gel, with homogeneity determined by staining. For certain purposes, high resolution will be desired and HPLC or  
20 a similar means for purification utilized. However, for most purposes, a simple chromatography column or polyacrylamide gel will be used to determine purity. Whether soluble or membrane bound, the present invention provides for substantially pure preparations. Various methods for their isolation from biological material may be devised, based in part upon the structural and functional descriptions contained herein. In  
25 addition, a protein that is chemically synthesized or synthesized in a cellular system that is different from the cell from which it naturally originates, will be substantially pure. The term is also used to describe receptors and nucleic acids that have been synthesized in heterologous mammalian cells or plant cells, *E. coli* and other prokaryotes.

As used herein, the terms "hybridization" (hybridizing) and "specificity" (specific for) in the context of nucleotide sequences are used interchangeably. The ability of two  
30 nucleotide sequences to hybridize to each other is based upon a degree of

complementarity of the two nucleotide sequences, which in turn is based on the fraction of matched complementary nucleotide pairs. The more nucleotides in a given sequence that are complementary to another sequence, the greater the degree of hybridization of one to the other. The degree of hybridization also depends on the conditions of stringency which include temperature, solvent ratios, salt concentrations, and the like. In particular, "selective hybridization" pertains to conditions in which the degree of hybridization of a polynucleotide of the invention to its target would require complete or nearly complete complementarity. The complementarity must be sufficiently high so as to assure that the polynucleotide of the invention will bind specifically to the target relative to binding other nucleic acids present in the hybridization medium. With selective hybridization, complementarity will be 90-100%, preferably 95-100%, more preferably 100%.

The present invention relates to a new purinergic receptor of the  $P_2$  subclass, which is referred to herein as the  $P_{2U2}$  receptor. Figure 1 shows the DNA sequence of the clone encoding the  $P_{2U2}$  receptor along with the deduced amino acid sequence. The amino acid sequence shown in Figure 1 includes four putative extracellular domains (the  $NH_2$ -terminus and ECD 1-ECD III) and seven putative transmembrane regions TM 1-TM VII). As used herein, the " $P_{2U2}$  receptor" refers to receptor in any animal species sharing a common biological activity with the human receptor contained in the clone described in Example 1 herein. This "common biological activity" includes but is not limited to an effector or receptor function or cross-reactive antigenicity. Using the native DNA encoding the human form of this receptor, the  $P_{2U2}$  receptors in other species, may be obtained.

Because the  $P_{2U2}$  receptor is activated by UTP, it is classified as a  $P_2$ -type purinergic receptor. Hydrophobicity/hydrophilic plots of the  $P_{2U2}$  receptor sequence shown in Figure 1 suggest that the  $P_{2U2}$  receptor has 7 putative transmembrane domains. This, along with the following characteristics, are consistent with characteristics that are observed in other  $P_2$ -type purinergic receptors:

- seven putative  $\alpha$ -helical transmembrane-spanning structures;
- amino terminus located on the extracellular side of the membrane;

carboxy terminus located on the intracellular side of the membrane; and conservation of sequence in the transmembrane spanning domains as compared with other  $P_2$ -purinergic receptors.

It has been found that the  $P_{2U2}$  receptor is expressed in many cell lines of megakaryocytic or erythroleukemic origin. In addition, the  $P_{2U2}$  receptor is expressed, at the RNA level, predominantly in the kidney. This receptor is unusual in that, although most purinergic receptors are present in the brain, the  $P_{2U2}$  receptor has not been found to be expressed in human brain tissue. The tissue distribution of the  $P_{2U2}$  receptor is described in Example 3.

Some  $P_2$  receptors have a strong preference for one nucleotide. Alternately, they may be activated by several nucleotides but the specificity for one nucleotide is usually an order of magnitude greater than for the other nucleotides. The  $P_{2U2}$  receptor is activated by ATP, ADP, UTP and UDP when expressed in *Xenopus* oocytes, with the following order of specificity:

UTP>UDP>ADP>ATP

However, unlike for other  $P_2$  receptors, the potency of ATP, ADP, UTP and UDP as agonists for the  $P_{2U2}$  receptor are close in value, with a mere five-fold difference:

One aspect of the present invention also relates to the human gene encoding the  $P_{2U2}$  receptor, which has both diagnostic and therapeutic uses as are described below.

Included within this invention are proteins or peptides having substantial homology with the amino acid sequence of Figure 1.

Ordinarily, the  $P_{2U2}$  receptors and analogs thereof claimed herein will have an amino acid sequence having at least 75% amino acid sequence identity with the  $P_{2U2}$  receptor sequence disclosed in Figure 1, more preferably at least 80%, even more preferably at least 90%, and most preferably at least 95%. Identity or homology with a sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the sequence of the  $P_{2U2}$  receptor, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. None

of the N-terminal, C-terminal or internal extensions, deletions, or insertions of the P<sub>2U2</sub> receptor sequence shall be construed as affecting homology.

Thus, the claimed P<sub>2U2</sub> receptor and analog molecules that are the subject of this invention include molecules having the P<sub>2U2</sub> receptor amino acid sequence; fragments thereof having a consecutive sequence of at least 10, 15, 20, 25, 30, or 40 amino acid residues from the P<sub>2U2</sub> receptor sequence of Figure 1; amino acid sequence variants of the P<sub>2U2</sub> receptor sequence of Figure 1 wherein an amino acid residue has been inserted N- or C-terminal to, or within, (including parallel deletions) the P<sub>2U2</sub> receptor sequence or its fragments as defined above; amino acid sequence variants of the P<sub>2U2</sub> receptor sequence of Figure 1 or its fragments as defined above which have been substituted by at least one residue.

P<sub>2U2</sub> receptor polypeptides include those containing predetermined mutations by e.g., homologous recombination, site-directed or PCR mutagenesis, and P<sub>2U2</sub> receptor polypeptides of other animal species, including but not limited to rabbit, rat, murine, porcine, bovine, ovine, equine and non-human primate species, and alleles or other naturally occurring variants of the P<sub>2U2</sub> receptor of the foregoing species and of human sequences; derivatives of the commonly known P<sub>2U2</sub> receptor or its fragments wherein the P<sub>2U2</sub> receptor or its fragments have been covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope); glycosylation variants of the P<sub>2U2</sub> receptor (insertion of a glycosylation site or deletion of any glycosylation site by deletion, insertion or substitution of appropriate amino acid); and soluble forms of the P<sub>2U2</sub> receptor. This invention also includes tagging the P<sub>2U2</sub> receptor, in particular for use in purification or diagnostic application. Types and methods of tagging are well known in the art, for example, the use of hexa-histidine tags.

Most sequence modifications, including deletions and insertions, and substitutions in particular, are not expected to produce radical changes in the characteristics of the P<sub>2U2</sub> receptor. However, when it is difficult to predict the exact effect of the sequence modification in advance of making the change, one skilled in the art will appreciate that the affect of any sequence modification will be evaluated by routine screening assays.



$P_{2U2}$  receptor peptides may be purified using techniques of classical protein chemistry, such as are well known in the art. For example, a lectin affinity chromatography step may be used, followed by a highly specific ligand affinity chromatography procedure that utilizes a ligand conjugated to biotin through the cysteine  
 5 residues of the ligand. Alternatively, a hexa-histidine tagged receptor may be purified using nickel column chromatography.

The nomenclature used to describe the peptide compounds of the invention follows the conventional practice where the N-terminal amino group is assumed to be to the left and the carboxy group to the right of each amino acid residue in the peptide. In  
 10 the formulas representing selected specific embodiments of the present invention, the amino- and carboxy-terminal groups, although often not specifically shown, will be understood to be in the form they would assume at physiological pH values, unless otherwise specified. Thus, the N-terminal  $H^+$  and C-terminal  $O^-$  at a physiological pH are understood to be present though not necessarily specified and shown, either in specific  
 15 examples or in generic formulas. Free functional groups on the side chains of the amino acid residues can also be modified by amidation, acylation or other substitution, which can, for example, change the solubility of the compounds without affecting their activity.

In the peptides shown, each gene-encoded residue, where appropriate, is represented by a single letter designation, corresponding to the trivial name of the amino  
 20 acid, in accordance with the following conventional list:

	<u>Amino Acid</u>	<u>One Letter Symbol</u>	<u>Three Letter Symbol</u>
25	Alanine	A	Ala
	Arginine	R	Arg
	Asparagine	N	Asn
	Aspartic acid	D	Asp
	Cysteine	C	Cys
30	Glutamine	Q	Gln
	Glutamic acid	E	Glu
	Glycine	G	Gly
	Histidine	H	His
	Isoleucine	I	Ile
35	Leucine	L	Leu
	Lysine	K	Lys

	Methionine	M	Met
	Phenylalanine	F	Phe
	Proline	P	Pro
	Serine	S	Ser
5	Threonine	T	Thr
	Tryptophan	W	Trp
	Tyrosine	Y	Tyr
	Valine	V	Val

10       The amino acids not encoded genetically are abbreviated as indicated in the discussion below.

          In the specific peptides shown in the present application, the L-form of any amino acid residue having an optical isomer is intended unless the D-form is expressly indicated by a dagger superscript (<sup>†</sup>). This invention also contemplates non-naturally occurring  
15       amino acids (typically those which are not naturally encoded) as are well known in the art.

          The compounds of the invention are peptides which are partially defined in terms of amino acid residues of designated classes. Amino acid residues can be generally subclassified into four major subclasses as follows:

          Acidic: The residue has a negative charge due to loss of H ion at physiological pH  
20       and the residue is attracted by aqueous solution so as to seek the surface positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH.

          Basic: The residue has a positive charge due to association with H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface  
25       positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH.

          Neutral/nonpolar: The residues are not charged at physiological pH, but the residue is attracted by aqueous solution so as to seek the outer positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium.  
30       These residues are also designated "hydrophobic" herein.

          Neutral/polar: The residues are not charged at physiological pH, but the residue is attracted by aqueous solution so as to seek the outer positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium.

It is understood, of course, that in a statistical collection of individual residue molecules some molecules will be charged, and some not, and there will be an attraction for or repulsion from an aqueous medium to a greater or lesser extent. To fit the definition of "charged", a significant percentage (at least approximately 25%) of the individual molecules are charged at physiological pH. The degree of attraction or repulsion required for classification as polar or nonpolar is arbitrary and, therefore, amino acids specifically contemplated by the invention have been classified as one or the other. Most amino acids not specifically named can be classified on the basis of known behavior.

Amino acid residues can be further subclassified as cyclic or noncyclic, and aromatic or nonaromatic, self-explanatory classifications with respect to the side chain substituent groups of the residues, and as small or large. The residue is considered small if it contains a total of 4 carbon atoms or less, inclusive of the carboxyl carbon. Small residues are, of course, always nonaromatic.

For the naturally occurring protein amino acids, subclassification according to the foregoing scheme is as follows:

Acidic: Aspartic acid and Glutamic acid

Basic/noncyclic: Arginine and Lysine

Basic/cyclic: Histidine

Neutral/polar/small: Glycine, serine and cysteine

Neutral/nonpolar/small: Alanine

Neutral/polar/large/nonaromatic: Threonine, Asparagine and Glutamine

Neutral/polar/large/aromatic: Tyrosine

Neutral/nonpolar/large/nonaromatic: Valine, Isoleucine, Leucine and Methionine

Neutral/nonpolar/large/aromatic: Phenylalanine, and Tryptophan

The gene-encoded secondary amino acid proline, although technically within the group neutral/nonpolar/large/cyclic and nonaromatic, is a special case due to its known effects on the secondary conformation of peptide chains, and is not, therefore, included in this defined group.

Certain commonly encountered amino acids, which are not encoded by the genetic code, include, for example, beta-alanine (beta-Ala), or other omega-amino acids, such as

3-amino propionic, 2,3-diamino propionic (2,3-diaP), 4-amino butyric and so forth, alpha-aminisobutyric acid (Aib), sarcosine (Sar), omithine (Om), citrulline (Cit), t-butylalanine (t-BuA), t-butylglycine (t-BuG), N-methylisoleucine (N-Melle), phenylglycine (Phg), and cyclohexylalanine (Cha), norleucine (Nle), cysteic acid (Cya) 2-naphthylalanine (2-Nal);  
5 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic);  $\beta$ -3-thienylalanine (Thi); and methionine sulfoxide (MSO). These also fall conveniently into particular categories.

Based on the above definitions,

Sar, beta-Ala, 2,3-diaP and Aib are neutral/nonpolar/small;

t-BuA, t-BuG, N-Melle, Nle, Mvl and Cha are neutral/nonpolar/large/

10 nonaromatic;

Orn is basic/noncyclic;

Cya is acidic;

Cit, Acetyl Lys, and MSO are neutral/polar/large/nonaromatic; and

Phg, Nal, Thi and Tic are neutral/nonpolar/large/aromatic.

15 The various omega-amino acids are classified according to size as neutral/nonpolar/small (beta-Ala, i.e., 3-aminopropionic, 4-aminobutyric) or large (all others).

Other amino acid substitutions of those encoded in the gene can also be included in peptide compounds within the scope of the invention and can be classified within this  
20 general scheme according to their structure.

All of the compounds of the invention, when an amino acid forms the C-terminus, may be in the form of the pharmaceutically acceptable salts or esters. Salts may be, for example,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{+2}$ ,  $\text{Mg}^{+2}$  and the like, the esters are generally those of alcohols of 1-6C.

25 In all of the peptides of the invention, one or more amide linkages (-CO-NH-) may optionally be replaced with another linkage which is an isostere such as -CH<sub>2</sub>NH-, -CH<sub>2</sub>S-, -CH<sub>2</sub>CH<sub>2</sub>-, -CH=CH- (cis and trans), -COCH<sub>2</sub>-, -CH(OH)CH<sub>2</sub>- and -CH<sub>2</sub>SO-. This replacement can be made by methods known in the art. The following references describe preparation of peptide analogs which include these alternative-linking moieties:

30 Spatola, Vega Data 1(3) "Peptide Backbone Modifications" (general review) (March

1983); Spatola, In "Chemistry and Biochemistry of Amino Acids Peptides and Proteins", B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983) (general review); Morley, J.S., *Trends Pharm Sci* (1980)pp. 463-468 (general review); Hudson, *et al.*, *Int J Pept Prot Res* (1979) 14:177-185 (-CH<sub>2</sub>NH-, -CH<sub>2</sub>CH<sub>2</sub>-); Spatola, *et al.*, *Life Sci* (1986) 5 38:1243-1249 (-CH<sub>2</sub>S-); Hann, *J Chem Soc Perkin Trans I* (1982) 307-314 (-CH-CH-, cis and trans); Almquist, *et al.*, *J Med Chem* (1980) 23:1392-1398 (-COCH<sub>2</sub>-); Jennings-White, *et al.*, *Tetrahedron Lett* (1982) 23:2533 (-COCH<sub>2</sub>-); Szelke, *et al.*, European Application EP 45665 (1982) CA:97:39405 (-CH(OH)CH<sub>2</sub>-); Holladay, *et al.*, *Tetrahedron Lett* (1983) 4:4401-4404 (-C(OH)CH<sub>2</sub>-); and Hruby, *Life Sci* (1982) 31:189-10 199 (-CH<sub>2</sub>S-).

The invention provides methods and materials useful in assay systems to determine the ability of candidate pharmaceuticals to affect the activity of the P<sub>2U2</sub> receptor. The isolation, recombinant production and characterization of the P<sub>2U2</sub> receptor allows for the design of assay systems using the P<sub>2U2</sub> receptor as a substrate and using agonists and 15 antagonists for the receptor as control reagents in the assay.

One embodiment of the invention relates to recombinant materials associate with the production of the P<sub>2U2</sub> receptor. These include transfected cells that can be cultured so as to display or express the P<sub>2U2</sub> receptor on its surface, thus providing an assay system for the interaction of materials with the native P<sub>2U2</sub> receptor where these cells or relevant 20 fragments of the P<sub>2U2</sub> receptor are used as a screening tool to evaluate the effect of various candidate compounds on the P<sub>2U2</sub> receptor activity *in vivo*, as is described below. Suitable cells include *Xenopus* oocytes and most mammalian cell lines.

Recombinant production of the P<sub>2U2</sub> receptor involves using a nucleic acid sequence that encodes the P<sub>2U2</sub> receptor, as is set forth in Figure 1, or its degenerate 25 analogs. The nucleic acids can be prepared either by retrieving the native sequence, as described below, or by using substantial portions of the known native sequence as a probe, or it can be synthesized *de novo* using procedures that are well known in the art.

The nucleic acid may be ligated into expression vectors suitable for the desired host and then transformed into compatible cells. Alternatively, nucleic acids may be 30 introduced directly into a host cell by techniques such as are well known in the art. The

cells are cultured under conditions favorable for the expression of the gene encoding the P<sub>2U2</sub> receptor and cells displaying the receptor on the surface are then harvested.

Suitable cells include *E. coli*, Chinese Hamster Ovary cells, human Jurkat T-cell line, and rat-2 fibroblast cell line, human astocytoma 1321N1 cell line and insect cell lines such as Sf-9.

This invention also relates to nucleic acids that encode or are complementary to a P<sub>2U2</sub> receptor polypeptide. These nucleic acids can then be used to produce the polypeptide in recombinant cell culture for diagnostic se or for potential therapeutic use. In still other aspects, the invention provides an isolated nucleic acid molecule encoding a P<sub>2U2</sub> receptor, either labeled or unlabeled, or nucleic acid sequence that is complementary to, or hybridizes under stringent conditions to, a nucleic acid sequence encoding a P<sub>2U2</sub> receptor. The isolated nucleic acid molecule of the invention excludes nucleic acid sequences which encode, or are complementary to nucleic acid sequences encoding, other known purinergic receptors which are not P<sub>2U2</sub> receptors such as the human P<sub>2U</sub>, and the chicken and bovine P<sub>2Y1</sub> receptors, and the like.

This invention also provides a replicable vector comprising a nucleic acid molecule encoding a P<sub>2U2</sub> receptor operably linked to control sequences recognized by a host transformed by the vector; host cells transformed with the vector; and a method of using a nucleic acid molecule encoding a P<sub>2U2</sub> receptor to effect the production of a P<sub>2U2</sub> receptor on the cell surface, comprising expressing the nucleic acid molecule in a culture of the transformed host cells and recovered from the cells. The nucleic acid sequence is also useful in hybridization assays for P<sub>2U2</sub> receptor-encoding nucleic acid molecules.

In still further embodiments of the invention, a method is described for producing P<sub>2U2</sub> receptors comprising inserting into the DNA of a cell containing the nucleic acid sequence encoding a P<sub>2U2</sub> receptor a transcription modulatory element (such as an enhancer or a silencer) in sufficient proximity and orientation to the P<sub>2U2</sub> receptor coding sequence to influence transcription thereof, with an optional further step comprising culturing the cell containing the transcription modulatory element and the P<sub>2U2</sub> receptor-encoding nucleic acid sequence.

This invention also covers a cell comprising a nucleic acid sequence encoding a  $P_{2U2}$  receptor and an exogenous transcription modulatory element in sufficient proximity and orientation to the above coding sequence to influence transcription thereof and a host cell containing the nucleic acid sequence encoding a  $P_{2U2}$  receptor operably linked to  
5 exogenous control sequences recognized by the host cell.

This invention provides a method for obtaining cells having increased or decreased transcription of the nucleic acid molecule encoding a  $P_{2U2}$  receptor, comprising: providing cells containing the nucleic acid molecule; introducing into the cells a transcription modulating element; and screening the cells for a cell in which the  
10 transcription of the nucleic acid molecule is increased or decreased.

$P_{2U2}$  receptor nucleic acids for use in the invention can be produced as follows. A  $P_{2U2}$  receptor "nucleic acid" is defined as RNA or DNA that encodes a  $P_{2U2}$  receptor, or is complementary to nucleic acid sequence encoding a  $P_{2U2}$  receptor, or hybridizes to such nucleic acid and remains stably bound to it under stringent conditions, or encodes a  
15 polypeptide sharing at least 75% sequence identity, preferably at least 80%, and more preferably at least 85% with the translated amino acid sequence shown in Figure 1. It is typically at least about 10 nucleotides in length and preferably has  $P_{2U2}$  receptor related biological or immunological activity. Specifically contemplated are genomic DNA, cDNA, mRNA and antisense molecules, as well as nucleic acids based on alternative  
20 backbone or including alternative bases whether derived from natural sources or synthesized.

"Stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015M NaCl/0.0015M sodium titrate/0.1% NaDodSO<sub>4</sub> at 50°C., or (2) employ during hybridization a denaturing agent such as  
25 formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50

µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C., with washes at 42°C. in 0.2 x SSC and 0.1% SDS.

"Isolated" nucleic acid will be nucleic acid that is identified and separated from contaminant nucleic acid encoding other polypeptides from the source of nucleic acid.

- 5 The nucleic acid may be labeled for diagnostic and probe purposes, using any label known and described in the art as useful in connection with diagnostic assays.

Of particular interest is a P<sub>2U2</sub> receptor nucleic acid that encodes a full-length molecule, including but not necessarily the native signal sequence thereof. Nucleic acid encoding full-length protein is obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if  
10 necessary, using conventional primer extension procedures to secure DNA that is complete at its 5' coding end. Such a clone is readily identified by the presence of a start codon in reading frame with the original sequence.

DNA encoding an amino acid sequence variant of a P<sub>2U2</sub> receptor is prepared as  
15 described below or by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of a P<sub>2U2</sub> receptor.

20 Techniques for isolating and manipulating nucleic acids are disclosed for example by the following documents: U.S. Patent No. 5,030,576 and International Patent Publications SO94/11504 and WO93/03162. See, also, Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989, and Ausubel, *et al.*, "Current Protocols in Molecular Biology," Vol. 2,  
25 Wiley-Interscience, New York, 1987.

As mentioned above, the availability of the isolated cells providing the P<sub>2U2</sub> receptor on their surface and the availability of the recombinant DNA encoding the P<sub>2U2</sub> receptor which permits display and expression of the receptor on host cell surfaces, all makes such as cells available as a valuable tool for evaluating the ability of candidate  
30 agonists or antagonists to bind to the receptor and thus contribute to the receptor's



activation or deactivation. In this manner, the invention is related to assay systems which utilize an isolated or a recombinantly produced  $P_{2U2}$  receptor to screen for agonist and antagonist activity of candidate drugs. This assay is especially useful in assuring that these candidate therapeutic agents have the desired effect of either activating or inhibiting the  $P_{2U2}$  receptor. Determination of these properties is essential in evaluating the specificity of drugs intended for binding other related receptors.

The host cells are typically animal cells, most typically mammalian cells. In order to be useful in the assays, the cells must have intracellular mechanisms which permit the receptor to be displayed on the cell surface. Particularly useful cells for use in the method of the invention are *Xenopus laevis* frog oocytes,, which typically utilize cRNA rather than standard recombinant expression systems proceeding from the DNA encoding the desired protein. Capped RNA (at the 5' end) is typically produced from linearized vectors containing DNA sequences encoding the receptor. The reaction is conducted using RNA polymerase and standard reagents. cRNA is recovered, typically using phenol/chloroform precipitation with ethanol and injected into the oocytes.

The animal host cells expressing the DNA encoding the  $P_{2U2}$  receptor or the cRNA-injected oocytes are then cultured to effect the expression of the encoding nucleic acids so as to produce the  $P_{2U2}$  receptor display on the cell surface. These cells then are used directly in assays for assessment of a candidate drug to bind, antagonize, or activate the receptor.

One method of evaluating candidates as potential therapeutic agents typically involves a binding assay in which the candidate (such as a peptide or a small organic molecule) would be tested to measure if, or to what extent, it binds the  $P_{2U2}$  receptor. Preferably, a mammalian or insect cell line is used to express the  $P_{2U2}$  receptor or plasma membrane preparations thereof, will be used in a binding assay. For example, a candidate antagonist competes for binding to the  $P_{2U2}$  receptor with either a labeled nucleotide agonist or antagonist. Varying concentrations of the candidate are supplied, along with a constant concentration of the labeled agonist or antagonist. The inhibition of binding of the labeled material can then be measured using established techniques. This

measurement is then correlated to determine the amount and potency of the candidate that is bound to the  $P_{2U2}$  receptor.

Another method of evaluating candidates for potential therapeutic applications typically involves a functional assay in which the candidate's effect upon cells expressing the recombinant  $P_{2U2}$  receptor is measured, rather than simply determining its ability to bind the  $P_{2U2}$  receptor. Suitable functional assays include those that measure calcium mobilization ( $^{45}\text{Ca}$  efflux or measurements of intracellular  $\text{Ca}^{+2}$  concentration with fluorescent dyes such as fura-2) and voltage clamp, described below.

For example, agonist-induced increases in  $^{45}\text{Ca}$  release by oocytes expressing cRNA encoding the  $P_{2U2}$  receptor or other mammalian recombinant cells producing the  $P_{2U2}$  receptor can be measured by the techniques described by Williams, *et al.*, *Proc Natl Acad Sci USA* (1988) 85:4949-4943. Intracellular calcium pools are labeled by incubating groups of 30 oocytes in 300  $\mu\text{l}$  calcium-free modified Barth's solution (MBSH) containing 50  $\mu\text{Ci } ^{45}\text{CaCl}_2$  (10-40  $\text{mCi/mg Ca}$ ; Amershal) for 4 hours at room temperature. The labeled oocytes or cells are washed, then incubated in MBSH II without antibiotics for 90 minutes. Groups of 5 oocytes are selected and placed in individual wells in a 24-well tissue culture late containing 0.5  $\text{ml/well}$  MBSH II without antibiotics. This medium is removed and replaced with fresh medium every 10 minutes; the harvested medium is analyzed by scintillation counting to determine  $^{45}\text{Ca}$  released by the oocytes during each 10-minute incubation. The 10-minute incubations are continued until a stable baseline of  $^{45}\text{Ca}$  release per unit time is achieved. Two additional 10-minute collections are obtained, then test medium including agonist is added and  $^{45}\text{Ca}$  release determined.

Using the above assay, the ability of a candidate drug to activate the  $P_{2U2}$  receptor can be tested directly. In this case, the agonists of the invention are used as controls. In addition, by using the agonists of the invention to activate the recombinant receptor, the effect of the candidate drug on this activation can be tested directly. Cells expressing the nucleic acids encoding the receptor are incubated in the assay in the presence of agonist with and without the candidate compound. A diminution in activation in the presence of the candidate will indicate an antagonist effect. Conversely, the ability of a candidate drug to reverse the antagonist effects of an antagonist of the invention may also be tested.

As indicated above, receptor activation can also be measured by means of the two-electrode voltage clamp assay. In this assay, agonist-induced inward chloride currents are measured in voltage-clamped oocytes that express the  $P_{2U2}$  receptor. The technique suitable for use in the instant invention is described by Julius, *et al.*, *Science* 5 (1988) 241:558-563.

The  $P_{2U2}$  receptor also has utility in assays for the diagnosis of renal system diseases and disorders by detection, in tissue samples, of aberrant expression of the  $P_{2U2}$  receptor.

Another aspect of the invention relates to  $P_{2U2}$  receptor agonists that imitate the 10 activated form of the  $P_{2U2}$  receptor. These agonists are useful as control reagents in the above-mentioned assays to verify the workability of the assay system. In addition, agonists for the  $P_{2U2}$  receptor may exhibit useful effects *in vivo* in treating kidney disease.

Another aspect of the invention relates to  $P_{2U2}$  receptor antagonists that are modified forms of  $P_{2U2}$  receptor peptides. Such antagonists bind to the  $P_{2U2}$  receptor, but 15 do not activate it, and prevent receptor activation by naturally occurring ligands by blocking their binding to the receptor. Another group of compounds within the scope of the invention are antagonists of the  $P_{2U2}$  receptor ligands, i.e., these are ligand inhibitors. Both these types of antagonists find utility in diminishing or mediating ligand-mediated events such as calcium release. Yet another second group of antagonists includes 20 antibodies designed to bind specific portions of the  $P_{2U2}$  receptor protein. In general, these are monoclonal antibody preparations which are highly specific for any desired region of the  $P_{2U2}$  receptor. The antibodies, which are explained in greater detail below, are also useful in immunoassays for the receptor protein, for example, in assessing successful expression of the gene in recombinant systems.

25 In both the agonists and antagonists, a preferred embodiment is that class of compounds having amino acid sequences that are encoded by the  $P_{2U2}$  receptor gene. Preferably, the agonists and antagonists have amino acid sequences, in whole or in part, corresponding to the extracellular domains of the  $P_{2U2}$  receptor. For example, preferred peptides of the invention correspond, in whole or in part, to either the amino terminus, 30 which is amino acid no. 1, methionine (M) to amino acid no. 23, lysine (K) (SEQ ID

NO:5); ECD I, which is amino acid no. 83, tyrosine (Y) to amino acid no. 99, arginine (R) (SEQ ID NO:6); ECD II, which is amino acid no. 162, asparagine (N) to amino acid no. 183, tyrosine (Y) (SEQ ID NO:7); or ECD III, which is amino acid no. 257, alanine (A) to amino acid no. 276, phenylalanine (F) (SEQ ID NO:8). Also included in the invention  
5 are isolated DNA molecules that encode these specific peptides. Accordingly, the invention pertains to isolated DNA molecules encoding human  $P_{2U2}$  receptor peptides comprising the amino acid sequence of Figure 1 from amino acid no. 1, methionine to amino acid no. 23, lysine (SEQ ID NO:5); from amino acid no. 83, tyrosine to amino acid no. 99, arginine (SEQ ID NO:6); from amino acid no. 162, asparagine to amino acid no.  
10 183, tyrosine (SEQ ID NO:7); and from amino acid no. 257, alanine to amino acid no. 276, phenylalanine (SEQ ID NO:8).

The invention also includes agonists and antagonists that affect receptor function by binding to one of the intracellular domains (ICDs) of the receptor. For example, preferred peptides within this aspect of the invention would correspond, in whole or in  
15 part, to either ICD I, which is amino acid no. 50, phenylalanine (F) to amino acid no. 60, isoleucine (I) (SEQ ID NO:11); ICD II, which is amino acid no. 120, arginine (R) to amino acid no. 141, leucine (L) (SEQ ID NO:12); ICD III, which is amino acid no. 208, tyrosine (Y) to amino acid no. 233, leucine (L) (SEQ ID NO:13); or to the carboxy terminus, which is amino acid no. 301, histidine (H) to amino acid no. 334, lysine (K)  
20 (SEQ ID NO:14). Also included in the invention are isolated DNA molecules that encode these specific peptides. Accordingly, the invention pertains to isolated DNA molecules encoding human  $P_{2U2}$  receptor peptides comprising the amino acid sequences of Figure 1 from amino acid no. 50, phenylalanine to amino acid no. 60, isoleucine (SEQ ID NO:11); amino acid no. 120, arginine to amino acid no. 141, leucine (SEQ ID NO:12); amino acid  
25 no. 208, tyrosine to amino acid no. 233, leucine (SEQ ID NO:13); and amino acid no. 301, histidine (H) to amino acid no. 334, lysine (K) (SEQ ID NO:14).

Also included are those compounds where one, two, three or more of the amino acid residues are replaced by one which is not encoded genetically. In other purinergic receptors, the third, sixth and seventh transmembrane ("TM") regions have been shown to  
30 play a role in ligand binding. See Erb, *et al. JBC* (1995) 270:4185-4188. Accordingly, it

is expected that the amino acid sequences of the TM III, TM VI and TM VII regions of the  $P_{2U2}$  receptor, in whole or in part, will be particularly useful in designed antibodies or peptides that can bind the receptor and block ligand binding.

The peptide agonists and antagonists of the invention are preferably about 10-100  
5 amino acids in length, more preferably 25-75 amino acids in length. These peptides can be readily prepared using standard solid phase or solution phase peptide synthesis, as is well known in the art. In addition, the DNA encoding these peptides can be synthesized using commercially available oligonucleotide synthesis instrumentation and recombinantly produced using standard recombinant production systems. Production using solid phase  
10 peptide synthesis is required when non-gene encoding amino acids are to be included in the peptide.

Another aspect of the invention pertains to antibodies, which have both diagnostic and therapeutic uses. Antibodies are able to act as agonists or antagonists by binding specific regions of the  $P_{2U2}$  receptor. The antibodies can be monoclonal or polyclonal, but  
15 are preferably monoclonal antibodies that are highly specific for the receptor and can be raised against the whole  $P_{2U2}$  receptor or regions thereof. Preferably, the antibodies are obtained by immunization of suitable mammalian subjects (typically rabbit, rat, mouse, goat, human, etc.) with peptides containing as antigenic regions those portions of the  $P_{2U2}$  receptor intended to be targeted by the antibodies. Critical regions include any region(s)  
20 of proteolytic cleavage, any segment(s) of the extracellular segment critical for activation, and the portions of the sequence which form the extracellular loops. These antibodies also find utility in immunoassays that measure the presence of the  $P_{2U2}$  receptor, for example in immunoassays that measure gene expression.

The antibodies of the present invention can be prepared by techniques that are well  
25 known in the art. Antibodies are prepared by immunizing suitable mammalian hosts in appropriate immunization protocols using the peptide haptens (immunogen) alone, if they are of sufficient length, or, if desired, or if required to enhance immunogenicity, conjugated to suitable carriers. The immunogen will typically contain a portion of the  $P_{2U2}$  receptor that is intended to be targeted by the antibodies. Critical regions include  
30 those regions corresponding to the extracellular domains of the  $P_{2U2}$  receptor protein.

Methods for preparing immunogenic conjugates with carriers such as bovine serum albumin, keyhole limpet hemocyanin, or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be effective; in other instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be desirable to provide accessibility to the hapten. The hapten can be extended at the amino or carboxy terminus with a cysteine residue or interspersed with cysteine residues, for example, to facilitate linking to carrier. The desired immunogen is administered to a host by injection over a suitable period of time using suitable adjuvants followed by collection of sera. Over the course of the immunization schedule, titers of antibodies are taken to determine the adequacy of antibody formation.

Polyclonal antibodies are suitable for many diagnostic and research purposes and are easily prepared. Monoclonal antibodies are often preferred to therapeutic applications and are prepared by continuous hybrid cell lines and collection of the secreted protein. Immortalized cell lines that secrete the desired monoclonal antibodies can be prepared by the method described in Kohler *et al.*, *Nature* (1975) 256:495-497 or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines are then screened by immunoassay techniques in which the antigen is the immunogen or a cell expressing the  $P_{2U2}$  receptor on its surface. Cells that are found to secrete the desired antibody, can then be cultured *in vitro* or by production in the ascites fluid. The antibodies are then recovered from the culture supernatant or from the ascites supernatant.

Alternately, antibodies can be prepared by recombinant means, i.e., the cloning and expression of nucleotide sequences or mutagenized versions thereof that at a minimum code for the amino acid sequences required for specific binding of natural antibodies. Antibody regions that bind specifically to the desired regions of receptor can also be produced as chimeras with regions of multiple species origin.

Antibodies may include a complete immunoglobulin or a fragment thereof, and includes the various classes and isotypes such as IgA, IgD, IgE, IgG1, IgG2a, IgG2b, IgG3 and IgM. Fragments include Fab, Fv,  $F(ab')_2$ ,  $Fab'$ , and so forth. Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant

portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as the Fab, Fab', or F(ab')<sub>2</sub> fragments is often preferable, especially in a therapeutic context, as these fragments have different immunogenicity than the whole immunoglobulin, and do not carry the biological activity of an immunoglobulin constant domain.

The antibodies thus produced are useful not only as potential agonist or antagonists for the receptor, filling the role of agonists or antagonist in the assays of the invention, but are also useful in immunoassays for detecting the activated receptor. As such these antibodies can be coupled to imaging agents for administration to a subject to allow detection of localized antibody to ascertain the position of P<sub>2U2</sub> receptors in either activated or unactivated form. In addition, these reagents are useful *in vitro* to detect, for example, the successful production of the P<sub>2U2</sub> receptor deployed at the surface of the recombinant host cells.

Yet another aspect of the invention relates to pharmaceutical compositions containing the compounds of the invention. The agonists and antagonists of the invention have therapeutic utility in (1) treating diseases caused by aberrant activation of this receptor in tissues where it is customarily found, for example in the kidney and (2) treating diseases whose symptoms can be ameliorated by stimulating or inhibiting the activity of the P<sub>2U2</sub> receptor.

The peptide agonists and antagonists of the invention can be administered in conventional formulations for systemic administration such as is well known in the art. Typical formulations may be found, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton PA, latest edition.

Preferred forms of systemic administration include injection, typically by intravenous injection. Other injection routes, such as subcutaneous, intramuscular, or intraperitoneal, can also be used. More recently, alternative means for systemic administration of peptides have been devised which include transmucosal and transdermal administration using penetrants such as bile salts or fusidic acids or other detergents. In addition, if properly formulated in enteric or encapsulated formulations, oral

administration may also be possible. Administration of these compounds may also be topical and/or localized, in the form of salves, pastes, gels and the like.

The dosage range required depends on the choice of peptide, the route of administration, the nature of the formulation, the nature of the patient's condition, and the judgment of the attending physician. Suitable dosage ranges, however, are in the range of 0.1-100 µg/kg of subject. Wide variations in the needed dosage, however, are to be expected in view of the variety of peptides available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization as is well understood in the art.

The invention also relates to the therapeutic, prophylactic and research uses of various techniques to block or modulate the expression of a  $P_{2U2}$  receptor by interfering with the transcription or translation of a DNA or RNA molecule encoding the  $P_{2U2}$  receptor. This includes a method to inhibit or regulate expression of  $P_{2U2}$  receptors in a cell comprising providing to the cell an oligonucleotide molecule which is antisense to, or forms a triple helix with,  $P_{2U2}$  receptor-encoding DNA or with DNA regulating expression of  $P_{2U2}$  receptor-encoding DNA, in an amount sufficient to inhibit or regulate expression of the  $P_{2U2}$  receptors, thereby inhibiting or regulating their expression. Also included is a method to inhibit or regulate expression of  $P_{2U2}$  receptors in a subject, comprising administering to the subject an oligonucleotide molecule which is antisense to, or forms a triple helix with,  $P_{2U2}$  receptor-encoding DNA or with DNA regulating expression of  $P_{2U2}$  receptor-encoding DNA, in an amount sufficient to inhibit or regulate expression of the  $P_{2U2}$  receptors in the subject, thereby inhibiting or regulating their expression. The antisense molecule or triple helix-forming molecule in the above methods is preferably a DNA or RNA oligonucleotide. These utilities are described in greater detail below.

The constitutive expression of antisense RNA in cells has been shown to inhibit the expression of about 20 different genes in mammals and plants, and the list continually grows (Hambor, *et al.*, *J Exp Med* (1988) 168:1237-1245; Holt, *et al.*, *Proc Natl Acad Sci* (1986) 83:4794-4798; Izant, *et al.*, *Cell* (1984) 36:1007-1015; Izant, *et al.*, *Science*



(1985) 229:345-352; and De Benedetti, *et al.*, *Proc Natl Acad Sci* (1987) 84:658-662.

Possible mechanisms for the antisense effect are the blockage of translation or prevention of splicing, both of which have been observed *in vitro*. Interference with splicing allows the use of intron sequences (Munroe, *EMBO J* (1988) 7:2523-2532 which should be less conserved and therefore result in greater specificity in inhibiting expression of a protein of one species but not its homologue in another species.

Therapeutic gene regulation is accomplished using the "antisense" approach, in which the function of a target gene in a cell or organism is blocked, by transfection of DNA, preferably an oligonucleotide, encoding antisense RNA which acts specifically to inhibit expression of the particular target gene. The sequence of the antisense DNA is designed to result in a full or preferably partial antisense RNA transcript which is substantially complementary to a segment of the gene or mRNA which it is intended to inhibit. the complementarity must be sufficient so that the antisense RNA can hybridize to the target gene (or mRNA) and inhibit the target gene's function, regardless of whether the action is at the level of splicing, transcription or translation. The degree of inhibition, readily discernible by one of ordinary skill in the art without undue experimentation, must be sufficient to inhibit, or render the cell incapable of expressing, the target gene. One of ordinary skill in the art will recognize that the antisense RNA approach is but one of a number or known mechanisms which can be employed to block specific gene expression.

By the term "antisense" is intended an RNA sequence, as well as a DNA sequence coding therefor, which is sufficiently complementary to a particular mRNA molecule for which the antisense RNA is specific to cause molecular hybridization between the antisense RNA and the mRNA such that translation of the mRNA is inhibited. Such hybridization must occur under *in vivo* conditions, that is, inside the cell. The action of the antisense RNA results in specific inhibition of gene expression in the cell. (See: Albers, *et al.*, Molecular Biology Of The Cell, 2nd Ed., Garland Publishing, Inc., New York, NY (1989), in particular, pages 195-196).

The antisense RNA of the present invention may be hybridizable to any of several portions of a target mRNA, including the coding sequence, a 3' or 5' untranslated region, or other intronic sequences. A preferred antisense RNA is that complementary to the

human P<sub>2U2</sub> receptor mRNA. As is readily discernible by one of skill in the art, the minimal amount of homology required by the present invention is that sufficient to result in hybridization to the specific target mRNA and inhibition of its translation or function while not affecting function of other mRNA molecules and the expression of other genes.

5 Antisense RNA is delivered to a cell by transformation or transfection with a vector into which has been placed DNA encoding the antisense RNA with the appropriate regulatory sequences, including a promoter, to result in expression of the antisense RNA in a host cell.

"Triple helix" or "triplex" approaches involve production of synthetic  
10 oligonucleotides which bind to the major groove of a duplex DNA to form a colinear triplex. Such triplex formation can regulate and inhibit cellular growth. See, for example: Hogan, *et al.*, U.S. Patent No. 5,176,996; Cohen *et al.*, *Sci Amer* (Dec. 1994) pp. 76-82; Helene, *Anticancer Drug Design* (1991) 6:569-584; Maher III, *et al.*, *Antisense Res Devel* (Fall 1991) 1:227-281; Crook, *et al.* eds., Antisense Research and Applications,  
15 CRC Press, 1993. It is based in part on the discovery that a DNA oligonucleotide can bind by triplex formation to a duplex DNA target in a gene regulatory region, thereby repressing transcription initiation (Cooney, *et al.*, *Science* (1988) 241:456). The present invention utilizes methods such as those of Hogan *et al.*, *supra* (incorporated herein by reference in its entirety), to designing oligonucleotides which will bind tightly and  
20 specifically to a duplex DNA target comprising part of the P<sub>2U2</sub> receptor-encoding DNA or a regulatory sequence thereof. Such triplex oligonucleotides can therefore be used as a class of drug molecules to selectively manipulate the expression of this gene.

Thus the present invention is directed to providing to a cell or administering to a subject a synthetic oligonucleotide in sufficient quantity for cellular uptake and binding to  
25 a DNA duplex of the target P<sub>2U2</sub> receptor-coding DNA sequence or a regulatory sequence thereof, such that the oligonucleotide binds to the DNA duplex to form a colinear triplex. This method is used to inhibit expression of the receptor on cells *in vitro* or *in vivo*. Preferably the target sequence is positioned within the DNA domain adjacent to the RNA transcription origin. This method can also be used to inhibit growth of cells which is  
30 dependent on expression of this receptor. The method may also be used to alter the

relative amounts or proportions of the P<sub>2U2</sub> receptor expressed on cells or tissues by administering such a triplex-forming synthetic oligonucleotide.

The following examples are intended to illustrate but not to limit the invention.

5

### Example 1

#### PCR (Polymerase Chain Reaction) Amplification of Related Purinergic Receptor cDNA with Degenerate Primers

DAMI cells (obtained from ATCC (#CRL9792)), were cultured in RPMI with  
10 10% fetal bovine serum, plus glutamine, penicillin/streptomycin and kanamycin, in 7%  
CO<sub>2</sub>/93% air and mRNA was isolated by the guanidine thiocyanate method. Poly-A(+) mRNA was selected two times using oligo-dT columns (Stratagene). The twice-selected poly-A+ mRNA was used to generate first-strand cDNA by priming with either oligo-dT or random primers and AMV reverse transcriptase (Invitrogen) as a template for PCR.  
15 Primers were designed based on the sequence of transmembrane region 3 (TM III, primer 3B) and transmembrane region 7 (TM VII, primer 7A2) from the mouse P<sub>2U</sub> (Lustig, *et al.*, *Proc Natl Acad Sci USA* (1993) 90:5113-5117) and the chicken P<sub>2Y1</sub> (Webb, *et al.*, *FEBS Letters* (1993) 324:219-225) receptor. The nucleotide sequence of 3B (SEQ ID NO:9) was:

20 5' A5(CT)CT(GTC)TT(CT)CTGAC(CTA)TG(CT)AT(CT)(AT)(GC)IGT(GTC)CA3'  
and the sequence for 7A2 (SEQ ID NO:10) was:

3' GG(GAT)(TC)A(CGA)(GA)AIAT(GA)AA(AG)(GA)AICGCC5'

where G is guanine, C is cytosine, A is adenine, T is thymidine and I is inosine, and the  
"()" indicate positions of degeneracy such that the sequences were a mixture with the  
25 indicated substitutions at that given position. The following conditions were used for  
PCR using Taq polymerase: 5 cycles of 93°C, 2 minutes; 60°C, 1.5 minutes; 72°C, 2.5  
minutes; 5 cycles of 93°C, 2 minutes; 55°C, 1.5 minutes; 72°C, 2.5 minutes; 25 cycles of  
93°C, 5 min. PCR products were purified over a size-selection column and ligated  
directly into the pCR2 TA cloning vector (Invitrogen) and the DNA was used to  
30 transform DH5α strain of *E. coli*. Colonies were selected and DNA was prepared for

restriction analysis and sequencing. Cycle sequencing was performed using Taq polymerase and dye-terminator mixes (Perkin-Elmer/ABI) and the results were analyzed on an ABI 373 automatic sequencer. Sequence results obtained with one clone, called 206.18, exhibited homology with published purinergic receptor sequences.

5

### Example 2

#### Isolation of Full-Length Human cDNA Encoding P<sub>2U2</sub>

Insert was isolated from the PCR clone of interest (206.18), purified from an agarose gel, radiolabeled with [ $\alpha$ -<sup>32</sup>P]dCTP(NEN) by random-priming (Stratagene), and used to screen a DAMI cDNA library in  $\lambda$ gt22. The library was generated using twice-selected poly-A<sup>+</sup> mRNA (see above) and first strand cDNA synthesis was primed with an oligo-dT primer and synthesized with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco/BRL). cDNA was directionally ligated into the SalI/NotI sites of the  $\lambda$ gt22 arms and packaged (Stratagene packaging extract) and amplified in the Y1090 (r-) strain. One million clones were screened at a density of 40,000/plate under the following conditions: duplicate nitrocellulose filters (S&S) were hybridized overnight at 42°C in a solution containing 50% deionized formamide, 5X SSC (sodium chloride, sodium citrate), 0.1 mg/ml heat denatured salmon sperm DNA, 0.1% sodium dodecylsulfate, 1X Denhardt's, 0.02M Tris, pH 7.5 and 1-2x10<sup>6</sup> cpm/ml of radiolabeled probe. Filters were washed twice at room temperature for 10 minutes in 0.1% sodium dodecylsulfate, 2X SSC and then at 55°C for 30 minutes in 0.2X SSC, 0.1% sodium dodecylsulfate, then exposed with an intensifying screen overnight at -70°C with Kodak XAR film. Positively hybridizing clones were plaque purified,  $\lambda$  DNA was prepared and the cDNA inserts were excised and subcloned into the commercially available pBluescript vector. The hybridizing and adjacent regions were sequenced on both strands as above on an ABI 373 automatic sequencer.

To isolate additional 5' sequence for the P<sub>2U2</sub> gene, a 5' proximal fragment from the largest DAMI clone (D8) was used to screen a Clontech human kidney cDNA library ( $\lambda$ gt10) under identical screening conditions as were used for the DAMI cDNA library. DAN from plaque-purified positively hybridizing clones from both libraries were analyzed

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by restriction digest. Inserts from clones of interest were excised and subcloned into the commercially available pBluescript vector and sequenced as above. The complete open reading frame as well as truncated versions of the full-length cDNA were cloned into *Xenopus* oocyte or mammalian expression vectors for functional analysis. The DNA sequence of the complete open reading frame for the longest cDNA isolated from the kidney cDNA library is shown in Figure 1 (SEQ ID NO:1). As shown in Figure 2, the deduced amino acid sequence of the P<sub>2U2</sub> cDNA shows extensive homology with other known purinergic receptors (Parr, *supra*, and Henderson, *supra*).

### Example 3

#### Expression of P<sub>2U2</sub> mRNA in Various Tissues and Cell Lines

Poly-(A)+ RNA was isolated from a variety of cell lines as described above. Five µg of each sample was denatured, electrophoresed on a 1.2% formaldehyde agarose gel, and transferred to nylon membrane. Blots were probed with [ $\alpha$ -<sup>32</sup>P]dCTP(NEN) labeled insert, as described for the library screenings, and hybridized at 42°C overnight in the following solution: 5X SSPE, 10X Denhardt's, 50% formamide, 2% sodium dodecylsulfate, 0.1 mg/ml heat denatured salmon sperm DNA. Blots were washed twice at room temperature for 15 minutes in 0.05% SDS, 2X SSC and then at 50°C for 30 minutes in 0.1X SSC, 0.1% SDS and exposed at -70°C to Kodak XAR film for 48-72 hours with an intensifying screen. Northern blots containing poly-A+ RNA from human tissues were purchased from Clontech and hybridized, washed and exposed as described above. Hybridization of RNA tissue blots with the labeled P<sub>2U2</sub> DNA fragment demonstrated that a 4.4 kB m RNA is abundantly expressed in human kidney, but is negative for other tissues examined (heart, brain, placenta, lung, liver, skeletal muscle, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocytes). These results distinguish this receptor from other reported purinergic receptors since these other receptors are abundant in brain. A series of mRNAs isolated from a variety of human hematopoietic and lymphocytic cell lines were used in a Northern analysis and a 4.4 kB message for the receptor was demonstrated to be abundant in

several cell lines of erythroleukemic (HEL, K562) and megakaryocytic (DAMI) origin, and not present in the monocytic cell line U937 or the T-cell derived Jurkat cell line.

#### Example 4

##### 5                    Demonstration of the Function of the Receptor in Oocytes

The native human receptor was produced in oocytes by cloning the 500 bp 5' truncation of the full-length kidney cDNA clone into the mammalian expression vector pcDNA3 (Invitrogen). Linearized DNA was used as a template for T7 polymerase (Ambion, Promega) for generation of capped *in vitro* transcribed mRNA following the  
10    supplier's specifications. Adult female *Xenopus laevis* were anesthetized in [0.015 g/l] 3-aminobenzoic acid ethyl ester for 10 minutes and 1 or 2 ovarian lobes were removed, followed by immediate suturing of the incisions. Oocytes were defolliculated at room temperature with collagenase (2 mg/ml) in Ca<sup>2+</sup>-free medium (OR-2) for 1-2 hr. Oocytes were stored at 18°C in ND-96 (96 mM sodium chloride, 2 mM potassium chloride, 1.8  
15    hydroxyethylpiperazine-N'[2-ethanesulfonic acid) with penicillin/streptomycin and injected with 50 nL RNA (1-2 µg/µl) 18-24 h after removal of the oocytes. Before recording, injected oocytes were stored at 18°C for 2-3 days with daily media changes.

A two-electrode voltage clamp (Axon Axoclamp2B) was used to measure agonist-induced currents from individual oocytes. Electrodes were pulled to resistances  
20    of 0.1-1 MΩ and filled with 3M KCl. Recordings were made at room temperature in ND96 from oocytes clamped at -70 mV using different agonist concentrations. Water-injected oocytes were used as a control. Figure 3 shows representative chloride currents obtained from oocytes injected with cRNA for the P<sub>2U2</sub> receptor and challenged with a variety of purinergic agonists (ADP, ATP, UTP, UDP).

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All references cited and mentioned above, including patents, journal articles and text, are all incorporated by reference herein, whether expressly incorporated or not.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters,

concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is  
5 intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth as follows in the scope of the appended claims.

**CLAIMS**

We claim:

1. An isolated and purified polypeptide comprising the amino acid sequence of Figure 1 (SEQ ID NO:2).

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2. An isolated and purified nucleic acid sequence encoding for the  $P_{2U2}$  receptor.

3. An isolated and purified nucleic acid sequence comprising the nucleotide sequence of Figure 1 (SEQ ID NO:1).

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ATAAAGTATG TTTAGCCCTC ATGTCACATG AACCTTTATG CATTGAAGAT	50
TGTTTCCCTT GCCCCCCCAG GGGGTGGGGT TATTTTCTA TCCTTGTTAA	100
CTTCCTATA TTATTATATA CACTTTGAGT TTTAGGGTAC ATGTGCACAA	150
AGTGCAGGTT AGTTACATAT GTATACATGT GCCATGTTGG TGTGCTGCAC	200
CCATTAACAC ATCATTTAGC ATGAGGTATA TCTCCTAATG TTATCCCTCC	250
CCCCTCCCC CACCCACAA CAGTCCCCGG AGTGTGATAT TCCCCTTTCC	300
TGTGTCCATG TGTTATTATT CCAATTCCCC ACCTATGAAG TGAAAATATG	350
CAGGTGTTTG GATTTTGTG CTTGGCAATA GTTTTGCTGA GAATGATGGT	400
TTCCAGCTTC ATCCATGTCC CTACAAAGGA CATGAACTCA TCATTTTTTA	450
TGACTGCATA GTATTCTATG GTGTATACAT GCCAACTTTT CTCCCCCCCC	500
TTTTTAAGCT CCTTCTTTCA CTGGCTTTCA TGATCCCACC AATTCCTGCT	550
TTTCCTTTT TGTTTTTTC TTCCAACAGA ATGGTTATGG TTAACTCAG	600
CAGAATTTGT TGAACAATA CGACATGCTG GGGATCATGG CATGGAATGC	650
M L G I M A W N A	
AACTTGCAAA AACTGGCTGG CAGCAGAGGC TGCCCTGGAA AAGTACTACC	700
T C K N W L A A E A A L E K Y Y L	
I	
TTTCCATTTT TTATGGGATT GAGTTCGTTG TGGGAGTCCT TGGAAATACC	750
S I F Y G I E F V V G V L G N T	
ATTGTTGTTT ACGGCTACAT CTTCTCTCTG AAGAACTGGA ACAGCAGTAA	800
I V V Y G Y I F S L K N W N S S N	
II	
TATTTATCTC TTAACTCT CTGTCTCTGA CTTAGCTTTT CTGTGCACCC	850
I Y L F N L S V S D L A F L C T L	
TCCCATGCT GATAAGGAGT TATGCCAATG GAACTGGAT ATATGGAGAC	900
P M L I R S Y A N G N W I Y G D	
GTGCTCTGCA TAAGCAACCG ATATGTGCTT CATGCCAACC TCTATACCAG	950
V L C I S N R Y V L H A N L Y T S	

FIG. 1A

III					
CATTCTCTTT	CTCACTTTTA	TCAGCATAGA	TCGATACTTG	ATAATTAAGT	1000
I L F	L T F I	S I D	R Y L	I I K Y	
ATCCTTTCCG	AGAACACCTT	CTGCAAAAGA	AAGAGTTTGC	TATTTTAATC	1050
P F R	E H L	L Q K K	E F A	I L I	
IV					
TCCTTGGCCA	TTTGGGTTTT	AGTAACCTTA	GAGTTACTAC	CCATACTTCC	1100
S L A I	W V L	V T L	E L L P	I L P	
CCTTATAAAT	CCTGTTATAA	CTGACAATGG	CACCACCTGT	AATGATTTTG	1150
L I N	P V I	T D N G	T T C	N D F A	
CAAGTTCTGG	AGACCCCAAC	TACAACCTCA	TTTACAGCAT	GTGTCTAACA	1200
S S G	D P N	Y N L I	Y S M	C L T	
V					
CTGTTGGGGT	TCCTTATTCC	TCTTTTGTG	ATGTGTTTCT	TTTATTACAA	1250
L L G	F L I P	L F V	M C F F	Y Y K	
GATTGCTCTC	TTCTAAAGC	AGAGGAATAG	GCAGGTTGCT	ACTGCTCTGC	1300
I A L	F L K Q	R N R	Q V A	T A L P	
CCCTTGAAAA	GCCTCTCAAC	TTGGTCATCA	TGGCAGTGGT	AATCTTCTCT	1350
L E K	P L N	L V I M	A V V	I F S	
VI					
GTGCTTTTAA	CACCCTATCA	CGTCATGCGG	AATGTGAGGA	TCGCTTCACG	1400
V L F T	P Y H	V M R	N V R I	A S R	
CCTGGGGAGT	TGGAAGCAGT	ATCAGTGCAC	TCAGGTCGTC	ATCAACTCCT	1450
L G S	W K Q Y	Q C T	Q V V	I N S F	
VII					
TTTACATTGT	GACACGGGCT	TTGGGCTTTC	TGAACAGTGT	CATCAACCCT	1500
Y I V	T R A	L G F L	N S V	I N P	
GTCTTCTATT	TTCTTTTGGG	AGATCACTTC	AGGGACATGC	TGATGAATCA	1550
V F Y F	L L G	D H F	R D M L	M N Q	
ACTGAGACAC	AAC TTCAAAT	CCCTTACATC	CTTTAGCAGA	TGGGCTCATG	1600
L R H	N F K S	L T S	F S R	W A H E	
AACTCCTACT	TTCATTGAGA	GAAAAGTGAG	GGGCTTGTGA	AACAGATTGT	1650
L L L	S F R	E K			
TCTACAGATG	AATCTGTAAG	CCAGTTACAG	TTTGCTTTAA	CTCATAGACA	1700
TCAATCAGAG	AGTGTACAG	ATTTAACCTT	GATCTAAAGA	CAAGTTGTAC	1750

FIG. 1B

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CCAGAGTATG TGAAAAGAAT GGGACGACAA GAATGTACTG GTTCTTCCT 1800  
CTAAGAATTG AAAGGAGTTG AACTGCCTTA TGTTTGGGCA TGTA ACTCCA 1850  
AAATACTAGG TAGTATAAGG CTTTCTCAAT CAGTCCCCAA ATGGAAGATA 1900  
TATAAAGCAA CAAGTTGTCT GCATTTGATC ACTGGTCAGA TTGTAAAAAA 1950  
AAAAAAAAAA AAGGGCGNCC GCCACCGCGG TGGAGCTCCA ATCGCC 1996

**FIG. 1C**

huP2U1	MAAD	-----	-----	GRND	IN	GTWDGDELGY	RCRFNE-DFK	32
bovP2Y1	TEVE	WPAVP	NGDTAFLAD	PGSP	GNSTV	TSTAASVSPF	KCALTKTGFG	50
huP2U2	LGIN	-----	-----	ADNA	CK	NWLAAEA	-----	23
huP2U1	YVLE	PVS	GV	VCV	GLCLNA	VGLYIFLCRL	KIMASTITM	82
bovP2Y1	FYY	PAY	IL	VET	ELGNS	YAINMFVHM	KPISGISVM	100
huP2U2	-YY	SIF	YGI	EFW	SVLGT	AVVGYIFSL	KVSSSNIM	72
huP2U1	AAS	LE	YVY	MARG	HPFS	TVCKLV	REFL	132
bovP2Y1	VLT	PA	LI	FNKT	DM	IFG	DAMCKLGRFI	150
huP2U2	LC	TE	PL	YAN	-GN	MYG	DVIGISNBYV	121
huP2U1	LGVL	R	RL	RWGR	RY	RR	VAGAVVLM	180
bovP2Y1	SG	AY	PL	GRL	KK	N	AVY	200
huP2U2	L	IKY	PREH	LLOK	KE	PI	IL	170
huP2U1	ACH	IS	AP	FSR	FV	SSV	MLGLL	230
bovP2Y1	AC	VD	IS	LSY	F	SMC	TTVAME	246
huP2U2	AC	VD	IS	PNYL	I	SMC	LTLG	218
huP2U1	TSG	GL	PR	SVRT	AV	VVL	AVL	277
bovP2Y1	KDL	DN	SP	STYL	MI	IVL	TV	296
huP2U2	RQV	AT	AL	PLNL	M	MAV	V	268
huP2U1	HTL	NA	N	MAV	MAV	MAV	MAV	326
bovP2Y1	AFN	DR	Y	ATY	QV	TR	GL	346
huP2U2	T	-QV	V	NSFY	IN	TR	GL	316
huP2U1	ATP	ARR	TL	GL	RR	SD	RT	375
bovP2Y1	EA	---	NLQ	---	SKSE	---	DM	373
huP2U2	LE	---	SFS	---	RW	HA	---	335

FIG. 2

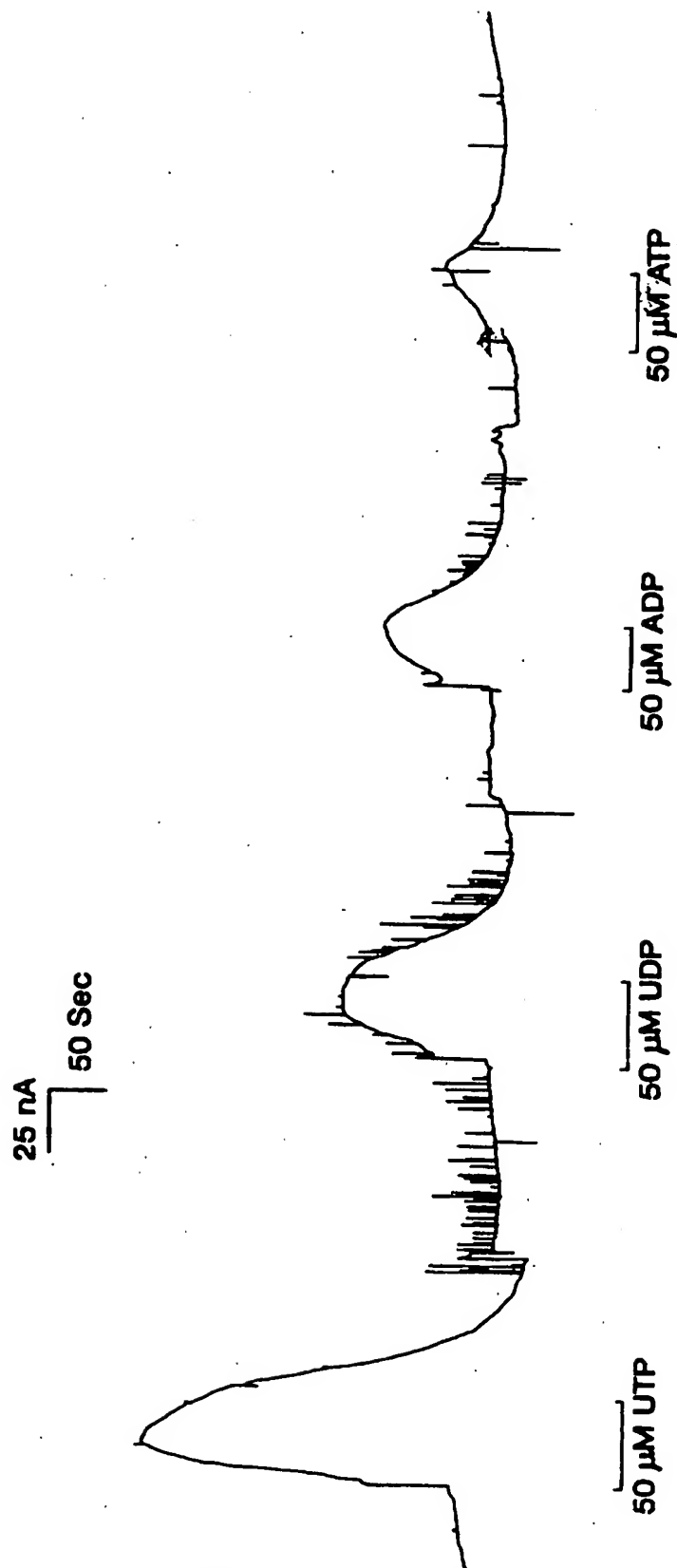


FIG. 3